

Maternal transfer of antibodies induced by infection with *Eimeria maxima* partially protects chickens against challenge with *Eimeria tenella*

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SUMMARY

Infection of breeding hens with *Eimeria maxima* induces production of *Eimeria*-specific IgG antibodies which are transferred to hatchlings via the egg yolk and confer a high degree of maternal immunity against homologous challenge and partial immunity to infection with another important species, *Eimeria tenella*. As an example, in an experiment using hatchlings from eggs collected between days 28 and 39 after infection of the hens with 20 000 sporulated *E. maxima* oocysts, control chicks (challenged with 100 sporulated oocysts) excreted 6.8 ± 1.2 million (mean \pm s.e., $n = 10$) or 5.8 ± 1.2 million ($n = 8$) oocysts of *E. maxima* or *E. tenella*, respectively, compared to 0.9 ± 0.4 million ($n = 5$) *E. maxima* oocysts or 2.2 ± 0.4 million ($n = 9$) *E. tenella* oocysts excreted by hatchlings of infected hens. This represents an 87% reduction in oocyst excretion with regard to *E. maxima* and a 62% reduction in oocyst excretion with regard to *E. tenella* in the progeny of the infected hens. In another experiment, eggs were collected from days 28 to 37 and again from days 114 to 123 after infection of the hens with *E. maxima* and hatchling oocyst excretion rates were 82% and 62%, respectively, reduced for *E. maxima* and 43% and 41%, respectively, reduced for *E. tenella* in the progeny of hens infected with *E. maxima* compared to the progeny of uninfected hens. ELISA and Western blot analyses of maternally-derived IgG revealed a high degree of cross-reactivity to antigens of *E. maxima* and *E. tenella*. Thus, maternally-derived, IgG-mediated cross-resistance to different species of *Eimeria* occurs in the chicken, most likely as a result of cross-recognition of conserved epitopes or proteins.

Key words: *Eimeria maxima*, *Eimeria tenella*, cross-protection, maternal immunity, IgG antibodies, Western blotting, ELISA.

INTRODUCTION

It is generally accepted that the immunity elicited by *Eimeria* species which infect the domestic chicken is highly specific as chickens completely immune to one species remain fully susceptible to infection with another (Wakelin & Rose, 1990). However, cross-species protection via maternally-derived immunity has not been investigated. Such an investigation would appear to be worthwhile for a combination of two reasons. First, it has been demonstrated that antibodies raised against one species of *Eimeria* cross-react with other species (Rose, 1987; Gilbert, Bhanushali & McDougald, 1988; Xie, Gilbert & McDougald, 1992) and, second, there is compelling evidence that maternal immunity to *Eimeria* is conferred by IgG antibodies. Thus, the egg yolk of a

normal chicken contains milligram quantities of IgG whereas there are only very small quantities of IgM or IgA in egg white (Lösch *et al.* 1986). Parasite-specific IgG levels in egg yolk or the sera of young chicks from hens infected with *Eimeria maxima* correlate very well with immunity to *Eimeria* in chicks but parasite-specific IgM or IgA antibodies are not detectable (Smith *et al.* 1994). It has also been demonstrated that gamma-livetin globulin fractions of egg yolks from infected hens are able to be injected subcutaneously into naive chickens and thereby protect them against infection (Rose, 1972). Thus, in this study we have tested the ability of antibodies induced by infection with *E. maxima* to cross-react with *E. tenella* and protect chickens against heterologous challenge via maternal transfer of IgG. Breeding hens were infected with *E. maxima*, the eggs collected and the hatchlings challenged with either *E. maxima* or *E. tenella* and oocyst excretion monitored to assess resistance. Furthermore, the antibodies present in the egg yolks were tested, by enzyme-linked immunosorbent assay (ELISA) and Western blotting, for reactivity to the two species.

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MATERIALS AND METHODS

Parasites and preparation of crude antigens

The Houghton strains of *E. maxima* and *E. tenella* were used throughout this study. Oocysts were passaged by oral inoculation (5000 sporulated oocysts) of 3 to 4-week-old female Lohmann Selected Leghorn chickens which were purchased on the day of hatching from Animalco, Stetten, Switzerland. Faeces were collected from day 6 to day 9 post-infection, and unsporulated and sporulated oocysts were purified by centrifugation, salt flotation and treatment with sodium hypochlorite as previously described (Wagenbach, Challey & Burns, 1966). For antigen preparation, the oocysts were suspended in an equal volume of phosphate-buffered saline (PBS) and an almost saturating quantity of 1 mm glass beads was added (for 1 mg of protein, approximately 10^8 oocysts are required). The oocysts were then vortexed with the glass beads for 5 min. Rupture of the oocysts was confirmed microscopically. Residual, unruptured sporocysts and sporozoites were lysed by three cycles of freeze/thawing (liquid nitrogen/40 °C) followed by sonication in a Branson sonifier at 20 watts for 20 s at 4 °C. The homogenate was then centrifuged at 12 000 *g* for 5 min and the supernatant fraction retained.

For the preparation of merozoites, chickens were infected at 3–4 weeks of age with 200 000 sporulated oocysts and at 96 h post-infection the chickens were killed under anaesthesia in accordance with Swiss animal protection regulations, their small intestines or caeca removed, slit open and washed in cold PBS. They were then cut into roughly 1 cm² pieces which were incubated for a maximum of 30 min at 40 °C in Hank's medium (Gibco, pH 7.6) containing 0.025 % trypsin (Difco), 1 % taurocholic acid (Fluka) and 10 mM MgCl₂. The large debris was then removed by filtering through gauze and, for *E. maxima*, smaller debris was removed by filtration through a 17 µm polymon mesh (Swiss Silk Bolting Cloth Manufacturing Company, Zurich). The merozoites were centrifuged at 1000 *g* for 10 min, the supernatant fraction discarded and the merozoites re-suspended in PBS. *E. maxima* merozoites were then filtered through a 10 µm mesh and the centrifugation and washing steps repeated 3 times. *E. tenella* merozoites were purified using a DEAE-52 column. Merozoite extracts were prepared by suspending merozoites in PBS at $1\text{--}2 \times 10^8$ /ml and freeze/thawing and sonication was done as described for the oocysts. Approximately 10^8 merozoites were recovered from each chicken.

For the preparation of gametocytes, chickens were infected with 10 000 oocysts at 4 weeks of age. At 136–138 h (*E. maxima*) or 143–145 h (*E. tenella*) post-infection, the chickens were killed, the guts removed and washed with cold SAC (170 mM NaCl,

10 mM Tris-HCl, pH 7, 10 mM glucose, 5 mM CaCl₂, 1 mM phenylmethanesulfonyl fluoride, 1 mg/ml bovine serum albumin). For *E. maxima*, one end of the intestine was tied with string and the gut filled with 0.5 mg/ml hyaluronidase in SAC. The other end of the intestine was then tied and the filled gut was placed in 37 °C PBS in a shaking water bath for 20 min. At the end of this incubation the intestines were slit open and the contents discarded. For *E. tenella*, the caeca were not tied but slit open and incubated at 37 °C in a beaker with hyaluronidase in SAC (20 caeca in 200 ml). The intestines or caeca were placed on top of a 17 µm mesh and the mucosa washed through with SAC (room temperature). The material left on the mesh was discarded and the flow through filtered through a 10 µm mesh. The gametocytes accumulated on the filter and were washed off with SAC and centrifuged at 800 *g* for 5 min. The gametocyte extract was made by incubating the gametocytes at room temperature for 30 min in 0.5 % deoxycholate in DEB (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 1 mM phenylmethanesulfonyl fluoride) – 2 million gametocytes/ml. The ruptured parasites were then centrifuged at 1500 *g* for 20 min and the supernatant fraction collected and dialysed against 10 mM phosphate buffer (pH 8) plus 1 mM phenylmethanesulfonyl fluoride at 4 °C overnight. The extract was then lyophilized and stored desiccated at –20 °C.

The protein concentrations of all extracts were determined using a BioRad protein determination kit.

Preparation of egg yolks for analysis by enzyme-linked immunosorbent assay (ELISA)

Eggs were opened into a Petri dish, the yolk membrane pierced and yolk fluid withdrawn with a Pasteur pipette. The yolk was mixed with an equal volume of PBS containing 0.3 % Tween 20, 0.02 % sodium azide and 0.05 % bovine haemoglobin (henceforth referred to as PBS-Tween). A small aliquot of this suspension was diluted to a final dilution of 1:500 with PBS-Tween and stored at –20 °C until use. (Titration experiments had shown that a dilution of 1:500 was optimal for analyses of large numbers of yolk samples.)

Preparation of sera for analysis by ELISA

Sera were obtained from blood collected by cardiac puncture of young chickens under anaesthesia. The blood was allowed to clot for 1 h at room temperature and then at 4 °C overnight before centrifugation, separation of sera, dilution (1:100) in PBS-Tween and storage at –20 °C. (Titration experiments had shown that a dilution of 1:1000 was optimal for analyses of large numbers of serum samples.)

Table 1. Protection against *Eimeria tenella* by maternally-derived antibodies from hens infected with *Eimeria maxima* (20000 sporulated oocysts/hen)(Results are means \pm S.E.)

Experiment no.*	Hatchling oocyst excretion ($\times 10^{-6}$)				Yolk IgG ELISA absorbance 405 nm†			
	<i>E. maxima</i>		<i>E. tenella</i>		Anti- unsporulated oocyst	Anti- sporulated oocyst	Anti- merozoite	Anti- gametocyte
	Control	Immunized	Control	Immunized				
1	6.8 \pm 1.2 (n = 10)	0.9 \pm 0.4 (n = 5)	5.8 \pm 1.2 (n = 8)	2.2 \pm 0.4 (n = 9)	0.58 \pm 0.07 (n = 10)	0.62 \pm 0.06 (n = 10)	0.81 \pm 0.12 (n = 10)	0.29 \pm 0.06 (n = 10)
2a	3.4 \pm 0.8 (n = 10)	0.6 \pm 0.3 (n = 10)	6.7 \pm 1.3 (n = 10)	3.8 \pm 0.6 (n = 10)	0.39 \pm 0.08 (n = 4)	0.93 \pm 0.07 (n = 4)	1.45 \pm 0.03 (n = 4)	0.40 \pm 0.06 (n = 4)
2b	5.7 \pm 0.7 (n = 10)	1.8 \pm 0.4 (n = 24)	3.7 \pm 0.6 (n = 10)	2.2 \pm 0.5 (n = 12)	0.26 \pm 0.03 (n = 16)	0.77 \pm 0.05 (n = 16)	0.79 \pm 0.06 (n = 16)	0.38 \pm 0.05 (n = 16)

* Experiment 1: eggs collected between days 28 and 39 after infection of the hens; Experiment 2a: eggs collected between days 28 and 37 after infection of the hens; Experiment 2b: eggs collected between days 114 and 123 after infection of the hens.

† For comparison, the following mean \pm S.E. ELISA absorbance values were recorded for over 50 control yolks: 0.05 \pm 0.01 for unsporulated oocyst, 0.09 \pm 0.01 for sporulated oocyst, 0.22 \pm 0.02 for merozoite and 0.06 \pm 0.01 for gametocyte.

ELISA

Soluble antigens were diluted in 0.1 M carbonate/bicarbonate buffer (pH 9.6) plus 0.02% sodium azide. Then 100 μ l aliquots of diluted antigens were allowed to absorb onto NUNC 'maxisorp' 96-well plates overnight at 4 °C. Unsporulated oocyst, sporulated oocyst and merozoite antigens were applied at 0.5 μ g/well whereas gametocyte antigen was applied at 1 μ g/well. The plates were then washed three times with 0.9% saline containing 0.3% Tween 20, prior to addition of 100 μ l aliquots of samples diluted in PBS plus 0.3% Tween 20, 0.02% sodium azide, and 0.05% bovine haemoglobin. After incubation for 120 min at 41 °C, plates were again washed three times and 100 μ l of diluted (1:2000) alkaline phosphatase-conjugated antibody to chicken IgG (Fc fragment specific, affinity purified, Bethyl Laboratories, Montgomery, Texas) was added to each well. After incubation at 41 °C for 120 min the plates were again washed three times and 100 μ l of a 1 mg/ml solution of *p*-nitrophenyl-phosphate (Sigma Chemical Ltd) in 0.05 M carbonate/bicarbonate (pH 9.8) was added to each well. After incubation at 37 °C for 30 min the reaction was stopped by addition of 50 μ l of 3 M NaOH to each well. Absorbance values were read at 405 nm in an automatic microelisa reader (MR610 autoreader, Dynatech Laboratories).

Western blotting

Antigens extracted from the various stages of development were separated on SDS-PAGE and Western transfer was performed as previously described (Wallach *et al.* 1989). Approximately 5–10 μ g of protein from unsporulated oocysts, sporulated oocysts, or merozoites, or 50 μ g gametocyte protein,

was electrophoresed in each lane (the use of a larger amount of gametocyte proteins was due to host contamination of this intracellular stage). These amounts were chosen according to the results of a titration experiment where the optimal amounts of the stage-specific proteins per lane were determined.

Immune or normal chicken sera or yolks were used to detect antigens on the Western blot as previously described (Wallach *et al.* 1989). The immune sera and yolks were used at dilutions of 1:100 and incubated for 2 h at room temperature.

Experimental design

Vedette broiler breeder hens and roosters were purchased from SEG, Zell, Switzerland at 18 weeks of age, since our surveys had shown that anti-*Eimeria* antibodies were much lower in pre-production hens than in those which had already been moved into the production area. The hens were kept in a manner which, as far as possible, mimicked commercial conditions. Thus, the hens were kept on the floor, in accordance with Swiss animal protection regulations, with 16 h of light/day to maximize egg production and were fed a strictly limited amount (100 g/bird/day) of non-medicated food (Nafag AG, Gossau, Switzerland). However, very high levels of hygiene were maintained and litter was changed 1–2 times/week to minimize the possibility of infection. Furthermore, the birds were housed at a much lower stocking density (6 hens plus 1 male/3 m² pen) than in commercial poultry houses and the litter was sampled (4 samples/pen of 7 animals) once/week to check for oocysts. Oocysts were only observed in the litter of groups experimentally infected with *E. maxima*. Egg production commenced when the hens were 23 weeks old and peak production was

reached by the time they were 30 weeks old. The hens were infected at this time.

In Experiment 1, a total of 13 hens was used. Six of these hens were infected with 20000 sporulated *E. maxima* oocysts and the other 7 hens were left uninfected. Eggs were collected between days 28 and 39 after infection of the hens and hatchlings were tested for maternally-derived immunity of *E. maxima* and *E. tenella*. A new group of hens was used in Experiment 2. Of these 6 were infected with 20000 *E. maxima* and 6 were left uninfected. Progeny of these hens were used on two occasions to test for maternally-derived immunity to *E. maxima* and *E. tenella*. Experiment 2a used hatchlings from eggs collected 28–37 days after infection of the hens and Experiment 2b used hatchlings collected 114–123 days after infection of the hens. Additional eggs were collected at various times after infection of the hens and sampled for antibody analysis. Hatchlings were challenged on day 3–4 post-hatching with 100 sporulated oocysts of *E. maxima* or *E. tenella*. This infective dose was chosen as a balance between the desire to use an inoculum which would reflect likely exposure levels encountered in the field and the need to use an infective dose which would ensure reproducible oocyst outputs. The chicks were housed in groups until the fifth day after infection, at which time they were individually caged. Faeces were collected from individually caged chicks daily from day 6 to day 9 post-infection and stored in 2% potassium chromate. The 4 day collection of faeces from each chick was homogenized in water to a final volume of 500 ml using a Waring blender. Samples of these homogenates were diluted 1:10 with saturated saline and duplicate subsamples were counted using a modified McMaster chamber slide. The duplicates were counted by separate people, neither of whom was aware from which group the sample originated.

RESULTS

Protection against Eimeria tenella by maternally-derived antibodies from hens infected with Eimeria maxima

The progeny of hens which had been infected with *E. maxima* were resistant to challenge with either *E. maxima* or *E. tenella* (Table 1). In Experiment 1, the mean oocyst excretion by hatchlings from infected hens challenged with *E. maxima* was reduced by, on average, 87% compared to the progeny of untreated hens. Two out of five chicks from infected hens did not excrete oocysts. In the same experiment, the oocyst excretion in hatchlings of infected hens challenged with *E. tenella* was 62% lower than that for control chicks. In Experiment 2a, *E. maxima*-challenged progeny of infected hens excreted, on average, 82% fewer oocysts (6 birds out of 10 did not excrete oocysts) than control chicks. Excretion of

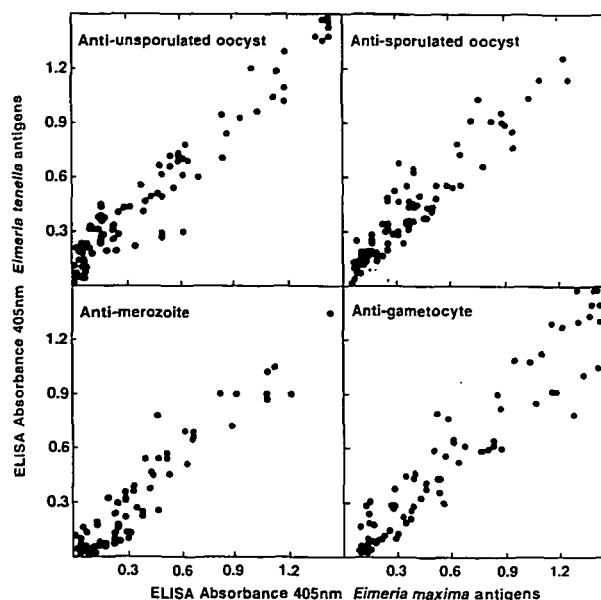


Fig. 1. ELISA cross-reactivity of sera, from hatchlings of hens infected with *Eimeria maxima*, with antigen extracts of *Eimeria tenella*.

oocysts of *E. tenella* was also reduced: oocyst excretion by the progeny of infected hens was 43% less than that of control chicks. Similar results were obtained in Experiment 2b. Thus, oocyst excretion by the progeny of infected hens was 68% (2 birds out of 24 did not excrete oocysts) and 41% reduced for challenge with *E. maxima* or *E. tenella*, respectively. In all the above cases, oocyst excretion was significantly lower in the progeny of infected hens ($P < 0.05$, Wilcoxon test statistic).

Parasite-specific IgG antibodies in yolks of hens infected with Eimeria maxima

Antibodies of the IgG isotype were readily detected, by ELISA, in the yolk of eggs from hens infected with *E. maxima* (Table 1 and Fig. 1). In all three of the protection trial experiments, the yolks of the eggs collected from the infected hens (Table 1) had significantly higher levels of *Eimeria*-specific antibody, for all the developmental stages tested, than yolks of the untreated hens ($P < 0.05$, one-way ANOVA, Student's *t*-test). Furthermore, yolk IgG reactivity to the *E. maxima* developmental stage extracts was very strongly correlated with reactivity to the corresponding *E. tenella* developmental stage extracts (Fig. 1; $r_s > 0.9$, $P < 0.0001$), with lines of best fit very close to linear ($y = 0.91x + 0.10$ for unsporulated oocysts; $y = 0.82x + 0.09$ for sporulated oocysts; $y = 0.92x - 0.01$ for merozoites; $y = 0.96x + 0.03$ for gametocytes).

Western blot analysis of protective sera

Sera were sampled from the chicks used in Experiment 2 on the day they were challenged with

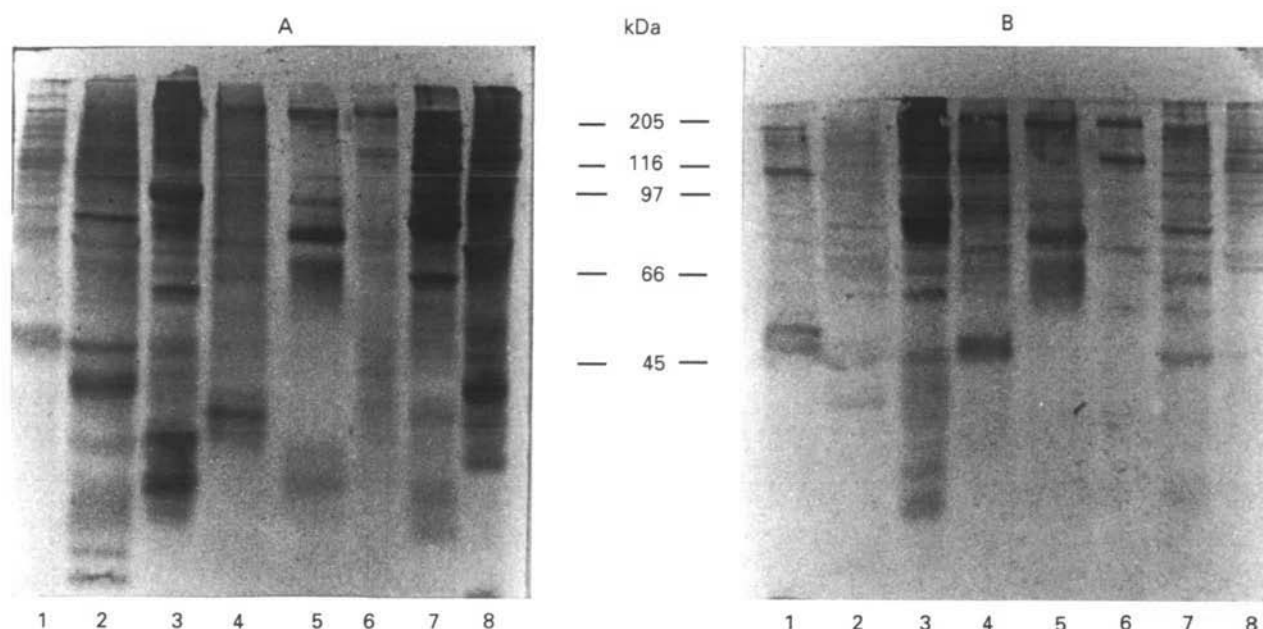


Fig. 2. Western blot cross-reactivity of sera from hatchlings of hens infected with *Eimeria maxima* with antigen extracts of *Eimeria tenella*. (Sera from control chicks were non-reactive and the blots are not reproduced here.) (A) Sera pool from 3-day-old chicks from Experiment 2a; eggs collected 28–37 days after infection of the hens; maternally-derived protection versus *E. maxima* = 82 %, protection versus *E. tenella* = 43 %. (B) Sera pool from 3-day-old chicks from Experiment 2b; eggs collected 114–123 days after infection of the hens; maternally-derived protection versus *E. maxima* = 68 %, protection versus *E. tenella* = 41 %. Lane 1: *E. tenella* gametocyte extract; Lane 2: *E. maxima* gametocyte extract; Lane 3: *E. tenella* merozoite extract; Lane 4: *E. maxima* merozoite extract; Lane 5: *E. tenella* sporulated oocyst extract; Lane 6: *E. maxima* sporulated oocyst extract; Lane 7: *E. tenella* unsporulated oocyst extract; Lane 8: *E. maxima* unsporulated oocyst extract.

Table 2. Approximate molecular weights of prominent proteins identified by Western blotting of developmental stages of *Eimeria maxima* and *Eimeria tenella*

	<i>Eimeria maxima</i>			<i>Eimeria tenella</i>			
Unsporulated oocyst	Sporulated oocyst	Merozoite	Gametocyte	Unsporulated oocyst	Sporulated oocyst	Merozoite	Gametocyte
190	230	230	82	210	230	230	135
160	175	175	56	140	95	95	60
150	—	—	40	85	80	80	—
110	—	—	—	70	—	65	—
105							
77							
75							
40							

E. maxima or *E. tenella* (that is, at 3 days of age). The sera from Experiment 2a (hatchlings from eggs collected 28–37 days after infection of the hens) were pooled to provide a sample for Western blotting representing a group of chicks that was ultimately well protected against infection with *E. maxima* (82 % reduction in oocyst excretion – Table 1). Similarly, the sera from Experiment 2b (hatchlings from eggs collected 114–123 days after infection of the hens) were pooled to provide a sample for Western blotting representing a group of chicks that

was ultimately only partially protected against infection with *E. maxima* (68 % reduction in oocyst excretion – Table 1). Both pools recognized numerous proteins in all developmental stages of both parasites (Fig. 2). The pool from the well-protected hatchlings reacted somewhat more strongly than the pool from the partially protected chicks, but the same proteins were prominent. The approximate sizes of the more prominent proteins are summarized in Table 2. Sera from 3-day-old progeny of uninfected hens did not react on Western blots.

DISCUSSION

Infection of breeding hens with *E. maxima* causes the production of copious quantities of parasite-specific IgG antibodies which are transferred via the egg yolk to hatchlings (Smith *et al.* 1994). As a consequence, hatchlings are extremely well protected against infection with *E. maxima*, some totally, as assessed by oocyst excretion (Rose, 1972; Smith *et al.* 1994). Progeny from hens infected with *E. maxima* are only partially protected against challenge with *E. tenella*. However, the level of hatchling immunity to challenge with *E. tenella* (41–62%; Table 1), induced by infection of the hens with *E. maxima*, is comparable to the levels of resistance reported by Rose & Long (1971), where progeny were protected by between 43 and 73% but only after the hens were infected on 4–5 occasions with *E. tenella*.

There are, in fact, a few reports of cross-resistance to *Eimeria* species in the chicken. For example, birds completely resistant to homologous challenge with high doses of *E. maxima* were also totally resistant to challenge with high doses of *Eimeria brunetti* as assessed by intestinal histology and faecal oocyst output (Rose, 1967*a*). Likewise, chickens resistant to homologous challenge with *E. brunetti* were highly resistant to challenge with *E. maxima*. Similarly, chickens infected with *Eimeria praecox* and subsequently challenged with *Eimeria acervulina* were relatively resistant to the heterologous species (Gore & Long, 1982). The converse experiment, in which birds were first infected with *E. acervulina* before being challenged with *E. praecox* also demonstrated the existence of cross-species immunity. There is also evidence of cross-resistance between *E. tenella* and *Eimeria necatrix* (Rose, 1967*b*). However, as pointed out by Rose (1987), these reports have not gained wide acceptance as being evidence for the existence of cross-species resistance because the results of the *E. maxima*/*E. brunetti* study were not reproducible (Hein, 1971) and the cross-protection observed in the other studies was relatively low, limited to certain developmental stages and apparently infective-site dependent. On the other hand, there are some examples where immunization with antigens derived from one species of *Eimeria* have induced a degree of cross-resistance (Danforth, Augustine & Jenkins, 1993).

The results of this study not only demonstrate the existence of cross-species resistance to *E. tenella* as a result of infection with *E. maxima*, but also provide an explanation for this phenomenon: ELISA and Western blot analyses reveal a large degree of cross-reaction of *E. maxima* and *E. tenella* antigens by yolk and hatchling sera resulting from infection of hens with *E. maxima*. Thus, ELISA absorbance values for all the developmental stages of the two parasites exhibit an almost perfect linear relationship. Western

blotting appears to confirm that *E. maxima* and *E. tenella* share antigenic epitopes. There are several proteins recognized in all stages of development in both the *Eimeria* species. However, the size of these proteins is generally different in the two. A notable exception to this is the 230 kDa protein band found in sporulated oocysts and merozoites of both species. This finding indicates that further investigation of this protein(s) as a potential candidate for inclusion in a maternally-based vaccine against coccidiosis, may be warranted.

In conclusion, the results presented here indicate that IgG antibody-mediated cross-resistance to different *Eimeria* species can occur. Furthermore, this cross-resistance may be the result of cross-recognition of conserved epitopes or proteins in different species. The implications of these results for vaccination against coccidiosis are enormous as they suggest that a limited number of highly conserved antigens administered as a maternally-based vaccine may protect broiler chicks against a variety of *Eimeria* species.

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